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ELIS JANAÍNA LIRA DOS SANTOS

ANÁLISE ULTRAESTRUTURAL DOS CEMENTÓCITOS SUBMETIDOS À APOSIÇÃO EXPERIMENTAL DO CEMENTO DENTAL

ULTRASTRUCTURAL ANALYSIS OF CEMENTOCYTES UNDER EXPERIMENTALLY-INDUCED DENTAL CEMENTUM APPOSITION

Piracicaba

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Dissertação de Mestrado apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Mestra em Clínica Odontológica, na Área de Periodontia.

Dissertation presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Master in Clinical Dentistry, in Periodontics area.

Orientador: Prof. Dr. Francisco Humberto Nociti Júnior

Coorientadora: Profa. Dra. Cristiane Ribeiro Salmon

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"A dor é inevitável, mas o sofrimento é opcional"

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RESUMO

Embora tenha havido avanço no entendimento da homeostase do cemento dental, o papel dos cementócitos permanece obscuro. Este estudo buscou caracterizar morfologica e ultraestruturalmente eventos relevantes nos componentes celulares do cemento dental durante a formação de novo cemento. Foi utilizado o modelo de indução de aposição do cemento dental nos molares inferiores de camundongos Swiss Webster por extração dos seus antagonistas. Para análise através de microscopia confocal os animais foram separados em um grupo induzido e um controle, e sacrificados após períodos de 6 e 21 dias (n=5). Foi mensurada a área de cemento dental na região apical dos grupos, além da quantidade de células nesta área delimitada, a relação célula/área e área do corpo dos cementótitos. Ademais, foi realizada uma análise morfológica da rede de canalículos. Posteriormente, animais do grupo induzido e controle foram eutanasiados após 21 dias de experimento (n=3), os cortes forma preparados e analisados por microscopia eletrônica de varredura (MEV). Finalmente, após 21 dias de experimento os animais (n=6) foram perfundidos com solução de Karnovsky e as mandíbulas foram processadas para análise por microscopia eletrônica de transmissão (MET). Os dados mostraram aumento na área do cemento dental no grupo induzido, comparado ao controle aos 21 dias ($p<0.05$), o que confirma a eficiência do modelo experimental na estimulação da aposição do cemento dental. No entanto, o número de cementócitos não foi alterado pela aposição de cemento, aos 21 dias foi identificada menor densidade celular na região estimulada ($p<0.05$). A área do corpo celular dos cementócitos foi significantemente aumentada frente à aposição aos 21 dias ($p<0.05$). As análise por microscopia eletrônica de transmissão demonstraram que, no grupo induzido, os cementócitos apresentaram corpos celulares e núcleos aumentados, além de maior quantidade de cromatima condensada na periferia e eucromatina espalhada no núcleo comparado ao controle. As análises por MEV revelaram dois padrões de lacunas no cemento celular: lacunas vazias e lacunas com remanescente celular, distribuidas irregularmente na matriz. Não foram observadas alteraçãoes organizacionais das fibras de Sharpey e dos feixes de fibras presentes na matriz. Desta forma, os achados morfológicos e ultraestruturais sugerem que os cementócitos, juntamente com cementoblastos, podem desempenhar um papel importante no controle da aposição experimental da matriz do cemento dental formado.

PALAVRAS-CHAVE: Cemento Dentário. Cementogênese. Microscopia.

ABSTRACT

Despite significant advance in understanding dental cementum homeostasis, the role cementocyte remais unclear. This study aimed at describing morphologically and ultrastructurally relevant events in extracellular matrix and cellular components in the course of new cementum development. Experimentally-induced dental cementum apposition model was used. Extraction of the maxillary molars of Swiss Webster was performed inducing mandibular molar supereruption. A morphological analysis of canaliculi network and cementocytes was performed in decalcified sections with confocal microscopy. Animals were assigned into two groups and sacrificed after 6 and 21 days (n = 5). Apical dental cementum area was measured, in addition the number of cells in this delimited area, the cell/ area ratio and body volume of the cementocytes. Sections on glass slides 20 μm (n = 3) were dehydrated, metallized and analyzed by scanning electron microscopy (SEM). Finally, after 21 days the animals (n = 6) were perfused with Karnovsky's solution and mandibles processed for transmission electron microscopy (MET) analysis. Data demonstrated increased cementum area in experimental group compared to control at 21 days ($p<0.05$) confirming experimental model efficiency regarding dental cementum apposition. However, cementocyte number has not been changed by cementum apposition, at 21 days a lower cell density was found in the area where DC apposition was induced ($p<0.05$). Cementocyte volume was significantly increased when DC apposition was induced at 21 days ($p<0.05$). TEM analysis showed that, for the EIA group, cementocytes presented larger body and nuclei sizes, more peripherally condensed chromatin and euchromatin spread in the nuclei than the controls. Furthermore, SEM analysis revealed that the two patterns observed at the cellular cementum were either lacunae with cell remnants or empty lacunae, with the present cementocytes occupying lacunae irregularly shaped and unevenly distributed into the matrix. Moreover, Sharpey's and matrix fiber bundles were well organized with no evidence of clear organizational changes between the groups (control versus EIA). Therefore, morphological and ultrastructural data suggest that cementocytes, together with cementoblasts, may play an important role in controlling experimental dental matrix apposition.

KEY WORDS: Dental cementum. Cementogenesis. Microscopy.

SUMÁRIO

1 INTRODUÇÃO

O cemento dental é um tecido mineralizado que cobre a superfície radicular do dente e promove por meio do ligamento periodontal, a inserção deste no osso alveolar (Bosshardt & Selvig, 1997; Foster & Somerman, 2012). Este tecido é composto por dois tipos celulares, os cementoblastos e os cementócitos, que tem origem nas células mesenquimais indiferenciadas do tecido conjuntivo do folículo dental durante a odontogênese (Bosshardt & Selvig, 1997). Os cementoblastos produzem a matriz extracelular por aposição de camadas. Durante o processo de cementogênese, enquanto muitos cementoblastos se alinham formando uma camada celular ao longo da superfície do cemento, alguns cementoblastos são aprisionados na matriz recentemente produzida e tornam-se cementócitos, a semelhança do tecido ósseo onde osteoblastos tornam-se osteócitos aprisionados. Da mesma forma que os osteócitos na matriz mineralizada do tecido ósseo, os cementócitos residem em lacunas no interior da matriz mineralizada do cemento dental e comunicam-se entre si e com os cementoblastos localizados na superfície do cemento por meio de prolongamentos citoplasmáticos que se estendem dentro de canalículos e canais (Bosshardt & Selvig, 1997; Bosshardt & Schroeder,1996; Saygin, Giannobile & Somerman, 2000) .

Por muitos anos, o papel dos osteócitos na manutenção da homeostasia do tecido ósseo foi subestimado ou até mesmo ignorado, uma vez que se acreditava que estas eram células inativas. Mais recentemente, vários grupos têm trabalhado no sentido de desvendar a real contribuição dos osteócitos na manutenção do equilíbrio do tecido ósseo (Dallas, Prideaux & Bonewald, 2013; Bellido, 2014). Trabalhos recentes, in vivo e in vitro, desvendaram muitas das funções suspeitas dos osteócitos, como mecano-sensorial e regulação da remodelação óssea e mineralização (Zhao et al., 2002; Feng et al., 2006; Gluhak-Heinrich et al., 2003; Kondoh et al., 2014; Lau et al., 2013). Além disso, estudos avançam em funções inovadoras dos osteócitos, tais como a capacidade de agir como um órgão endócrino, sugerindo um potencial para influenciar células e tecidos muito além do microambiente ósseo (Dallas, Prideaux & Bonewald, 2013).

Alguns estudos sugerem que a sinalização entre osteócitos e a paratireoide e rim indica a importância da função endócrina do osteócitos e o impacto que a manutenção da viabilidade dos osteócitos pode ter em outros tecidos (St John et al., 2014; Ito et al., 2015; Lindberg et al., 2014).

Neste contexto, o entendimento da participação de importantes vias de sinalização, como a via Wnt, e da secreção de moléculas chave no processo de remodelação do osso como a esclerostina (Compton & Lee, 2014; Honasoge, Rao & Rao, 2014; Sapir-Koren & Livshits, 2014) e o fator de crescimento 23 de fibroblastos (FGF23) (Lindberg et al., 2014; Sapir-Koren & Livshits, 2014) e da proteína de matriz dentinária (DMP1) (Feng et al., 2003; Toyosawa et al., 2012; Lee, Yamaguchi & Iimura, 2014) principalmente por osteócitos, foram os avanços recentes mais relevantes na biologia do esqueleto.

Diferente do tecido ósseo, o cemento não possui um papel de "*turnover*" ou remodelação significativos, ou seja, é capaz de reparar-se a um grau limitado e não é reabsorvido em condições normais (Bosshardt & Selvig, 1997; Foster & Somerman, 2012). Além disso, o cemento responde diferentemente a intervenções terapêuticas nos casos em que os tecidos periodontais são perdidos como consequência da doença periodontal, onde a regeneração do cemento é frequentemente difícil e imprevisível (Bosshardt & Sculean, 2009). Estudos têm sido conduzidos no sentido de tentar identificar fatores específicos do cento dental (Foster et al., 2007; Foster et al., 2011; Foster et al., 2012; Salmon et al., 2013; Nociti & Somerman, 2013) e na compreensão dos seus processos fisiológicos que possam contribuir com o desenvolvimento de técnicas regenerativas mais confiáveis e previsíveis. Entretanto, o conhecimento a nível celular e molecular do cemento dental, tanto durante a cementogênese ou como no tecido maduro em condições fisiológicas normais ou alteradas ainda está pobremente definido (Saygin, Giannobile & Somerman, 2000). Evidências recentes sugerem que cementócitos não apenas dividem características morfológicas com osteócitos, mas também expressam inúmeros reguladores do metabolismo de tecidos mineralizados em comum. Foi demonstrado que, similarmente aos osteócitos, cementócitos expressam a esclerostina (Jager et al, 2010; Lehnen et al., 2012; Kuchler et al., 2014) e em estudo com animais "*knockout*" ambos os fenótipos de osso e

cemento foram alterados (Kuchler et al., 2014). Ainda, paralelamente ao que ocorre com osteócitos, importantes reguladores do metabolismo de tecidos mineralizados, incluindo DMP1, são expressos por cementócitos e podem ser regulados via hormônio 1,25(OH)2D3 (1,25D) (Nociti et al., 2014) e Fgf23 (Chu et al., 2010). Assim como osteócitos, cementócitos expressam fatores importantes na regulação do metabolismo do fosfato (Pi), incluindo proteína de anquilose progressiva (ANK), e animais *"knockout"* para essa proteína desenvolvem fenótipos alterados em tecidos ósseos e dentais (Foster et al., 2011; Nociti et al., 2002).

Embora seja sugerido que cementoblastos, juntamente com células do ligamento periodontal e do osso alveolar, são essenciais para a manutenção da homeostasia dos tecidos periodontais (Saygin, Giannobile & Somerman, 2000; Hefti, 1993; Bartold, 1995; Grzesik & Narayanan, 2002), o papel dos cementócitos nesse processo permanece completamente desconhecido. Dadas as características morfológicas e biológicas comuns para osteócitos e cementócitos, além das similaridades entre a matriz do tecido ósseo e do cemento dental, nossa hipótese é de que cementócitos possam exercer um papel significativo para a manutenção não apenas do cemento dental, mas também de todo o conjunto que compõe o periodonto de sustentação. E apesar de anos de pesquisa e desenvolvimento de conhecimento sobre o papel do cemento, o qual acredita-se ser importante no processo reparativo após a doença periodontal, ainda há aspectos relevantes em relação a este tecido que permanecem não esclarecidos.

Desta forma, o presente estudo foi realizado utilizando-se técnicas celulares de alta resolução para fornecer informações sobre a morfologia e ultraestrutura dos cementócitos, com o objetivo de determinar o papel exercido pelos cementócitos na homeostasia dos tecidos periodontais. O melhor conhecimento sobre o perfil celular dos cementócitos poderá fornecer novos "*insights*" sobre o tecido que possam permitir o desenvolvimento de terapias eficientes e mais previsíveis para regeneração dos tecidos periodontais.

2 ARTIGO

ULTRASTRUCTURAL ANALYSIS OF CEMENTOCYTES UNDER EXPERIMENTALLY-INDUCED DENTAL CEMENTUM APPOSITION

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ABSTRACT

Background: Cementocytes not only share morphological features with osteocytes, but also express several common biomarkers. Nevertheless, in contrast to osteocytes, there is a lack of evidence on the potential role of cementocytes in tissue homeostasis. Here, it is hypothesized that cementocytes are key players in dental cementum biology. **Material and Methods**: This study used a model of experimentally induced dental cementum apposition (EIA) in mice to determine whether cementocytes are responsive cells in such environment. Mandibular 1st molars were randomly induced to erupt for 6 and 21 days after extracting opposing maxillary molars, whereas contralateral teeth were used as controls. Histological sections were prepared from the 1st molars' region for transmission and scanning electron microscopy (TEM and SEM, respectively), whereas non-decalcified sections were labeled with fluorescein isothiocyanate and observed under a confocal microscope. Quantitative data was submitted to two-way ANOVA (α=5%) followed by Tukey's test (α=5%). **Results:** Data analysis showed an increased area of dental cementum (DC) for the EIA group compared to control at 21 days ($p<0.05$), and therefore, confirmed DC apposition. Intriguingly, as cementocyte's number was not affected by DC apposition, at 21 days a lower cell density was found in the area where DC apposition was induced ($p<0.05$). Cementocyte volume was significantly increased when DC apposition was induced at 21 days ($p<0.05$). TEM analysis showed that, for the EIA group, cementocytes presented larger body and nuclei sizes, more peripherally condensed chromatin and euchromatin spread in the nuclei than the controls. Furthermore, SEM analysis revealed that the two patterns observed at the cellular cementum were either lacunae with cell remnants or empty lacunae, with the present cementocytes occupying lacunae irregularly shaped and unevenly distributed into the matrix. Moreover, Sharpey's and matrix fiber bundles were well organized with no evidence of clear organizational changes between the groups (control versus EIA). **Conclusion:** Together, these findings provide new insights on DC biology and reveal cementocytes as potential targets for tissue engineering applications.

KEY WORDS: dental cementum, cementogenesis, ultrastructure

INTRODUCTION

Dental cementum (DC) is a “bonelike” mineralized connective tissue covering the root surface of teeth serving as the attachment structure for periodontal ligament (1). Both, DC and bone, present a highly similar structure and composition with a comparable mineral-to-organic matrix ratio (2). Nonetheless, mature bone has a lamellar organization and actively participates in the metabolism of the body, providing a reservoir for calcium and other elements (3-6); whereas DC is not innervated, avascular, less cellular and exhibits little or no remodeling, but continues to grow in thickness throughout life (1, 7-9). DC is composed of two cells types, cementoblasts and cementocytes, both originated from mesenchymal stem cells during odontogenesis. Cementoblasts have been described as large and cuboidal cells with euchromatin-rich nuclei, plentiful rough endoplasmatic reticulum (RER) and Golgi apparatus, whereas cementocytes reside in lacunae and exhibit canalicular network and irregular spacing (10).

The hallmarks of periodontal disease are the destruction of soft connective tissues, bone loss, and loss of connective tissue attachment to DC; these alterations, if left untreated, may lead to tooth loss (11-13). The aim of periodontal therapy is to regenerate and restore the various periodontal components affected by disease. New therapeutic approaches available include the use of barrier membranes for guided tissue regeneration, and applying growth factors and enamel matrix proteins to root surfaces (14-16); however, the effectiveness of these approaches is not predictable, especially when considering new cementum and attachment formation. A significant barrier to improved therapies for regeneration is that cementum biology remains poorly understood. Fundamental questions centered on cementocytes include whether they are physiologically active cells, whether they remain in communication with cells in the periodontal ligament (PDL) region, whether they are involved in cementum formation and repair, including response of cementum to altered demands of tooth use, and whether they regulate osteoclast activity at the cementum surface. Recent studies suggest that cementocytes, apparently terminally differentiated cells, present an *in vivo* expression profile that parallels that of osteocytes, including the expression of dentin matrix protein 1 (DMP1), sclerostin (SOST), E11/gp38, tumor

necrosis factor receptor superfamily, member 11b (osteoprotegerin/OPG), and tumor necrosis factor superfamily member 11 (RANKL) (17-20). In addition, using the experimentally-induced apposition (EIA) model to induce DC apposition, followed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) proteomic analysis, our group provided initial evidence that cementocytes may have an active role in neocementogenesis (21). Although, some progress has been made in terms of improving comprehension on the biology of DC, it is presently unknown whether cementocytes have *in vivo* functions related to cementum formation or repair. Here, it was asked whether cementocytes are dynamic actors in neocementogenesis capable of undergoing morphological and ultrastructural changes as a response to the continuous apposition of DC in a mouse model.

MATERIALS AND METHODS

Animals and surgeries: Animal procedures were performed at the Piracicaba Dental School Animal Facility in accordance with the guidelines of the University Committee for Ethics in Animal Research (Protocol#3788-1). The experimentally-induced apposition model (EIA) was used to promote dental cementum (DC) apposition, as previously proposed (22, 23). Briefly, male Swiss Webster mice (35 days old) were sedated and the right/left maxillary molars were randomly extracted to allow DC apposition on the antagonist mandibular 1st molars. The contralateral side was used as the control group. After 6 and 21 days (21) of DC apposition, 19 animals were euthanized by cervical dislocation, the mandibles dissected and placed either in paraformaldehyde 4% for confocal imaging, formalin 4% for scanning electron microscopy (SEM) and Karnovsky's solution for transmission electron microscopy (TEM).

Fluorescence analysis by confocal microscopy: In order to access changes in cementocyte morphology as a consequence of the process of continuous neocementogenesis, undecalcified samples were labeled with fluorescein isothiocyanate (FITC) and analyzed by confocal microscopy. Experimentally-induced DC apposition was performed and the animals euthanized after 6 and 21 days (n=5). After euthanize, mandibles were dissected, fixed in 4% paraformaldehyde at 4°C under agitation for 24 hours, dehydrated in 70% ethanol for 24 hours, and embedded in SamplKwick Fast Cure Acrylic resin (Buehler, Lake Bluff, Illinois, USA), following the manufacturer's instructions. A diamond low-speed saw (South Bay Technology, San Clemente, California, USA) was used to obtain 400-500 µm bucco-lingual sections from the mesial root of the 1st mandibular molar, and then manually ground to a final thickness of approximately 300 µm. Sections were dehydrated in graded ethanol series (50%, 70%, 90% for 10 minutes each, and 100% for 30 minutes), and stained with 1% fluorescein isothiocyanate (FITC) for 4 hours at room temperature protected from light. Next, samples were washed in 100% ethanol for 30 minutes, let to dry at room temperature for 2 hours and mounted on Entellan®. The cellular cementum was

immediately analyzed in a confocal microscope with the laser set to 488 nm (Leica, Wetzlar, Hessen, Germany). Six serial images equally distant (0.8 µm), representative of the whole tooth, were obtained from Z-stack by confocal laser scanning microscopy (CLSM) in the bucco-lingual direction for five animals. Cellular cementum area, cementocyte number and density were determined, by a blind and calibrated examiner (EJLS), on the mesial root of the mandibular 1st molar for both, control and EIA groups, using an image-analysis system (Image J – Java-based image processing software package v3.91, National Institutes of Health (NIH), Bethesda, Maryland, USA). In addition, dimensional changes were accessed in twelve randomly selected cementocytes according to its location: close to dentin (n=6) and close to the periodontal ligament (n=6). Histological measurements were calculated as an average value for each animal and a final average determined for each experimental group. Figure 1 schematically illustrates all the accessed parameters.

Scanning Electron Microscopy (SEM): SEM analyses were performed on hemi-mandibles from mice under EIA and controls (n=3). At 21 days, animals were sacrificed, mandibles were dissected, fixed in 4% neutral formalin for 48 h and subsequently demineralized in 10% EDTA solution at 4°C under agitation for 17 days. Bucco-lingual paraffin serial sections (20 µm) were obtained from the mesial root of the 1st mandibular molar. Next, sections were dehydrated (3x, xylene), air-dried for 10 minutes at room temperature, dried to the critical point and gold coated (Denton Vacuum Desk II, Moorestown, New Jersey, USA). Images were obtained and analyzed in a scanning electron microscope (JEOL JSM 5600LV, Tokyo, Japan) operating at 15 kV in electron mode. A blinded examiner (EJLS) performed all the SEM analysis.

Transmission Electron Microscopy (TEM): In order to perform TEM analysis, animals under EIA and control (n=6) were perfused with Karnovsky's fixative solution (4% paraformaldehyde, 2% glutaraldehyde in Sorensen phosphate buffer 0.1M, pH 7.4) at 21 days after EIA. Mandibles were dissected and post-fixed in Karnovisky's solution for 24 hours, washed in PBS, decalcified in 10% EDTA for 17 days. Next, samples were

washed in PBS, post-fixed in 1% osmium tetroxide (OsO_4), washed in PBS, dehydrated in a graded acetone series (75%, 95%, 100%) and embedded in LR White resin (EMS, Hatfield, Pennsylvania, USA). Thin sections (70-90 nm) were obtained from mesial root of 1st mandibular molars in a bucco-lingual direction, transferred to carbon-coated. Formvar grids, stained with uranyl acetate (10 minutes) followed by lead citrate (2 minutes). Mesh grids were then viewed and imaged on a transmission electron microscope JEM 1400 (Jeol, Japan). A blinded examiner (EJLS) performed all the TEM analysis.

Statistical analysis: DC area, cell number, cell density and dimensional changes were statistically compared between EIA and control groups using the two-way ANOVA followed by the Tukey's test (α=0.05) and Shapiro–Wilk test (SAS system for Windows v9.4 - SAS Institute, Cary, NC, USA).

RESULTS

Confocal analysis: CLSM allowed qualitative and quantitative assessments. Qualitative analysis focused on the cementocytes' canalicular system, whereas quantitative parameters included the impact of EIA on cellular cementum area, cementocyte number, cellular cementum density (*e.g.,* number of cementocyte cellular / cellular cementum area) and cementocyte body size (figure 1). Data analysis showed that the cellular cementum area was increased by EIA at 21 days ($p<0.05$), whereas at 6 days, no clear effect of EIA was found on the area of cellular cementum ($p>0.05$) (Figures 2A-C). Intragroup analysis revealed that the number of cementocytes was increased over time for both groups ($p<0.05$); however, intergroup comparisons showed that there was no difference between the experimental groups at 6 and 21 days (Figure 3A). Therefore, as cellular cementum area was significantly increased by EIA at 21 days and cementocytes number was not affected, cellular density was significantly decreased by EIA at 21 days (Figure 3B). Since cellular dimensional changes have been reported as an important characteristic of metabolic active cells, we examined whether

cementocytes' body size was affected in response to an experimental neocementogenesis model (EIA). Intragroup analysis showed that cementocytes' body size was significantly increased in response to continuous neocementogenesis ($p<0.05$), whereas in the control group, cementodytes' body size decreased overtime ($p<0.05$). Furthermore, at 21 days, intergroup analysis demonstrated that EIA resulted in larger cementocytes as compared to the control group (Figure 4A-B). In the current study, we found no relevant qualitative differences either when control and EIA groups were compared or when cementocytes and osteocytes were comparatively analyzed (Figure 5).

Scanning Electron Microscopy (SEM) findings: In the present investigation, SEM analysis was also used to qualitatively examine the impact of EIA on cementocytes at 21 days. Overall, for both experimental groups, dental cementum consisted of masses of radially arranged, extrinsic Sharpey's fibers having surfaces covered by numerous intrinsic collagen fibers. These Sharpey's fibers maintained a perpendicular orientation to the cementum surface and emerged with the fibers of the periodontal ligament. Sharpey's and matrix fiber bundles were well organized with no evidence of clear organizational changes between the groups (control versus EIA). Moreover, SEM analysis revealed that the two patterns observed at the cellular cementum were either lacunae with cell remnants or empty lacunae, with the present cementocytes occupying lacunae irregularly shaped and unevenly distributed into the matrix. Figure 6 illustrates the representative SEM findings.

Transmission Electron Microscopy (TEM) findings: In the present study, TEM analysis was employed to explore potential changes on the cellular ultrastructure of cementocytes in response to the process of neocementogenesis. We found that cementocytes from both, control and EIA groups presented defined nuclei, intact nuclear membrane and relatively poorly developed rough endoplasmic reticulum (RER). Cementocytes from the EIA group featured increased cell and nuclei volume, more peripherally condensed chromatin and also and euchromatin spread in the nuclei

than the controls. Surprisingly, for the EIA experimental group, there was a trend towards a larger empty space between the cementocyte and its lacunae.

In the current investigation, we used this system to also direct our analyses to the cementoblasts. In general, for the EIA group, cementoblasts were identified as cells with abundant cytoplasm, nuclei with prominent nucleoli and rough endoplasmic reticulum (RER) with large number of minute granular particles (ribosomes) attached to the cisternae surface, which represents the typical cell morphology of high metabolic rate, indicating intense protein synthesis. In comparison with cementocytes, cementoblasts were larger cells presenting well-defined nuclei, intact nuclear membrane and well-developed RER (Figure 7).

DISCUSSION

Dental cementum (DC), a mineralized tissue that covers the tooth root, is similar in several aspects to bone, however DC biology remains poorly understood. Previously thought to be passive cells residing within the bone matrix, currently osteocytes are accepted as active modulator of bone homeostasis and remodeling, and have been reported to actively contribute to endocrine regulation of mineral metabolism (4, 24). The panorama of what is known about DC comes from studies on its histology and composition (25, 26, 27), and although recent progress has been made (19, 21), there is still a lack of information related to the role of cementocytes in DC biology. Due to advances in the exploration of osteocytes as an important actor in bone metabolism (28, 29), comprehensive research on cementocytes has been encouraged in order to clarify the complete function of these cells in periodontal homeostasis and regeneration. Here, it was used the EIA model associated with *in vivo* fluorescent imaging, TEM and SEM analysis to test the hypothesis that cementocytes are active cells engaged with neocementogenesis. Our findings indeed demonstrate a number of evidences that cementocytes might react to continuous DC apposition, including increasing cementocytes' body size, increasing nuclei volume and chromatin

condensation distinctions. To the best of our knowledge, this is the first time that a potential role of cementocytes in neocementogenesis has been demonstrated.

Sicher and Weinmann's historic model of the unopposed rodent molar established the biological foundation for root cementum and bone growth as consequence of physiological movement. The authors suggested removing the upper molars on one side and allowing the lower molars to erupt without an antagonist. This model seems to be useful to study aspects of tissue remodeling during axial tooth movement (30, 31).

Authors have revealed that new cementum and alveolar bone are formed at the apex of the un-opposed molar after 12 days (32), therefore in the present study time points at 6 and 21 days were considered according to previous study using the same approach (21). In earlier periods initial molecular changes occur while in the late periods perhaps morphological changes have been occurred. Eruption and distal movement induced by unloading are result of distal bone resorption and mesial/apical bone apposition (22) that is the reason to evaluate mesial root in the present study.

A number of approaches have been used to determine whether distinct cell types are engaged with biological processes. As we have noted in previous ultrastructural analysis using TEM, cementoblasts are located nearby to external cementum layer and present evidences of metabolically active cells such as pronounced rough endoplasmic reticulum (RER). Cementocytes are irregular spacing in cellular cementum and differ widely in shape, some being elongated, more stellate or cuboidal, with relatively few organelles compared to cementoblasts (1, 27, 33). An important event in alveolar bone is the change from osteoblast to osteocyte, wherein cell morphology is substantially modified. Osteoblasts undergoes morphological change from polygonal to a more stellate shape with dendritic processes, once enclosed in bone tissue features a polarity to direct mineral formation (29). While osteocyte is embedded and differentiates, is observed cellular body contraction as well as reduction in the cytoplasmic organelles such as Golgi complex and endoplasmic reticulum (34, 35). Cementoblast to cementocytes transition demonstrate change in cellular shape and size, as well as decrease in cytoplasmic organelles, indicative of low metabolic rate (30). Indeed, in this study, experimental and control groups indicated some ultrastructural differences regardless of cementoblasts, which feature extensive

rough endoplasmic reticulum (RER) under stimuli, indicating increased cellular activity. Cementocytes also presented changes as larger cells and nuclei volume under experimentally-induced apposition, however RER are not extremely developed compared to cementoblasts. These findings may suggest that cementocytes does not produce protein similar to cementoblasts but may be participating in signaling for more active matrix apposition by cementoblasts.

Previous Scanning electron microscopy analysis was used to view transverse sections of mandibular 1st molar under EIA and revealed that the area of cellular cementum was increased at the root apex by 37% (32). In addition, our findings demonstrate that cellular cementum area was significantly increased by EIA at 21 days and cementocytes number was not affected. It was hypothesize, although cementocytes number has not been affected by EIA, cementocytes present at cellular cementum play an active role on neocementogenesis and these findings suggest EIA model affect cementocyte body size instead cementocyte number. Some authors have shown that increased cell volume is associated with cellular activity (36, 37). Overall, the ultrastructural results of this study suggest that experimentally-induced dental cementum apposition model may promote changes in the cellular machinery once the cellular volume is increased and this is not related to increase cementocytes in number.

Osteocyte reside within a ellipsoid and regular lacunae, their dendritic connections provide communication between osteocytes and surface cells like osteoblasts and osteoclasts as well as with distant cells via signaling (38, 39). These long cytoplasmic processes radiate in different directions surround cell body. This presents significant value to overcome the matrix enclosed environment and to establish communication with other cells as well as access nutrients and oxygen (4, 40). Analysis using SEM have demonstrated that similar to osteocytes, cementocytes occupy lacunae and display projections within a canalicular network, suggesting they are adapted as cells embedded within a extracellular mineralized matrix, establishing communication system with one another and with cells outside the cellular cementum (4, 10, 41).

Although studies have shown that osteocytes embedded in mineralized bone matrix play an important role in bone tissue homeostasis, the role of cementocytes in dental cementum has not been thoroughly elucidated in terms of associated cells and

regulatory factors involved in pathological processes and other conditions. Periodontal disease, for instance, refers to the inflammatory processes that occur in the tissues surrounding the teeth in response to periodontopathogenic microorganisms (42, 43), and neutrophils, macrophages and lymphocytes play important role in the host local response (44). Moreover, immunoinflammatory response includes events as activation of complement system, antigen presentation to T lymphocytes, antibodies production and cytokines and chemokines expression (45, 46, 47). Tumor necrosis factor (TNF-α), Interleukin-1β (IL-1β) and Interleukin-6(IL-6) are the main cytocines expressed. TNF-α regulate adhesion molecules and stimulate the production of new chemokines, matrix metalloproteinases and RANKL. IL-1β and IL-6 are associated with migration of inflammatory cells and osteoclastogenesis (48, 49, 50). Moreover, osteoclastogenesis mechanism in periodontal disease is associated to these regulatory molecules. Osteoblasts express RANKL, the main regulator of osteoclast differentiation and activation, osteoclast cells induce bone resorption (51, 52). On the other hand, the inhibitor of osteoclastogenesis, osteoprotegerin (OPG) upon binding to the RANK receptor, present in the preosteoclasts, block bone resorption (53, 54). Recently, osteocytes have been considered the major source of RANKL and may have greater capacity to support osteoclastogenesis (55). Bone lacunar canalicular system seems to conduct bone remodeling and resorption, transmitting signals to coordinate osteoblast and osteoclast activity (56, 57). *In vivo* experimental model in mice for apical periodontitis demonstrated bone resorption and as the disease progresses RANKL/OPG expression has been increased. In addition, periodontal ligament and cementum reabsorption has been detected. Overall, the study suggest cementocytes express RANKL in response to endodontic infection and may be involved in the pathogenesis of apical periodontitis (20). DC and bone, present a highly similar structure and composition, the fact that cementocytes express RANKL may suggest these cells play an active role in modulate development of periodontal disease. Similar to osteocyte, cementocytes also express Sclerostin (SOST) (18), DMP1 (58) and important factors in the regulation of phosphate metabolism (Pi), including progressive ankylosis protein (ANK) (59, 60). Therefore, these findings strongly suggest that cementocytes are involved in events such as the development of resorption and

perhaps cell recruitment to inflammatory sites, due to their similarity to osteocytes. However, further studies have been encouraged to prove this important role.

A similar study using the same model of experimentally-induced apposition (EIA) in mice and the same time points (6 and 21 days) identified proteins associated with neocementogenesis. These encompassed SerpinF1, tenascin-X (TNX), aspirin (ASPN), collagen type XI alpha 1 (COL11A1), osteonectin (SPARC), thrombospondin 1 (THBS1), osteopontin (OPN), and fibromodulin (FMOD). Overall, proteomic analysis demonstrated significantly altered protein profile in dental cementum under EIA. The study demonstrated the model efficiency similar to our findings (21).

Recently, with the establishment of improved therapies for periodontal tissues, regenerative approach following periodontal disease it has become one of the main goals in periodontal therapy. Future studies focusing on defining the mechanisms involved in cementogenesis should assist in establishing the significance of cementocytes in modulating cementum formation and clarify their effects on the periodontium. Our findings indicate that cementum is responsive to experimentally-induced apposition model, due to an increased area of dental cementum (DC) after stimuli. Ultrastructural changes including increasing cementocytes' body size, increasing nuclei volume and chromatin condensation suggest that cementocytes may play active role in dental cementum homeostasis.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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FIGURES

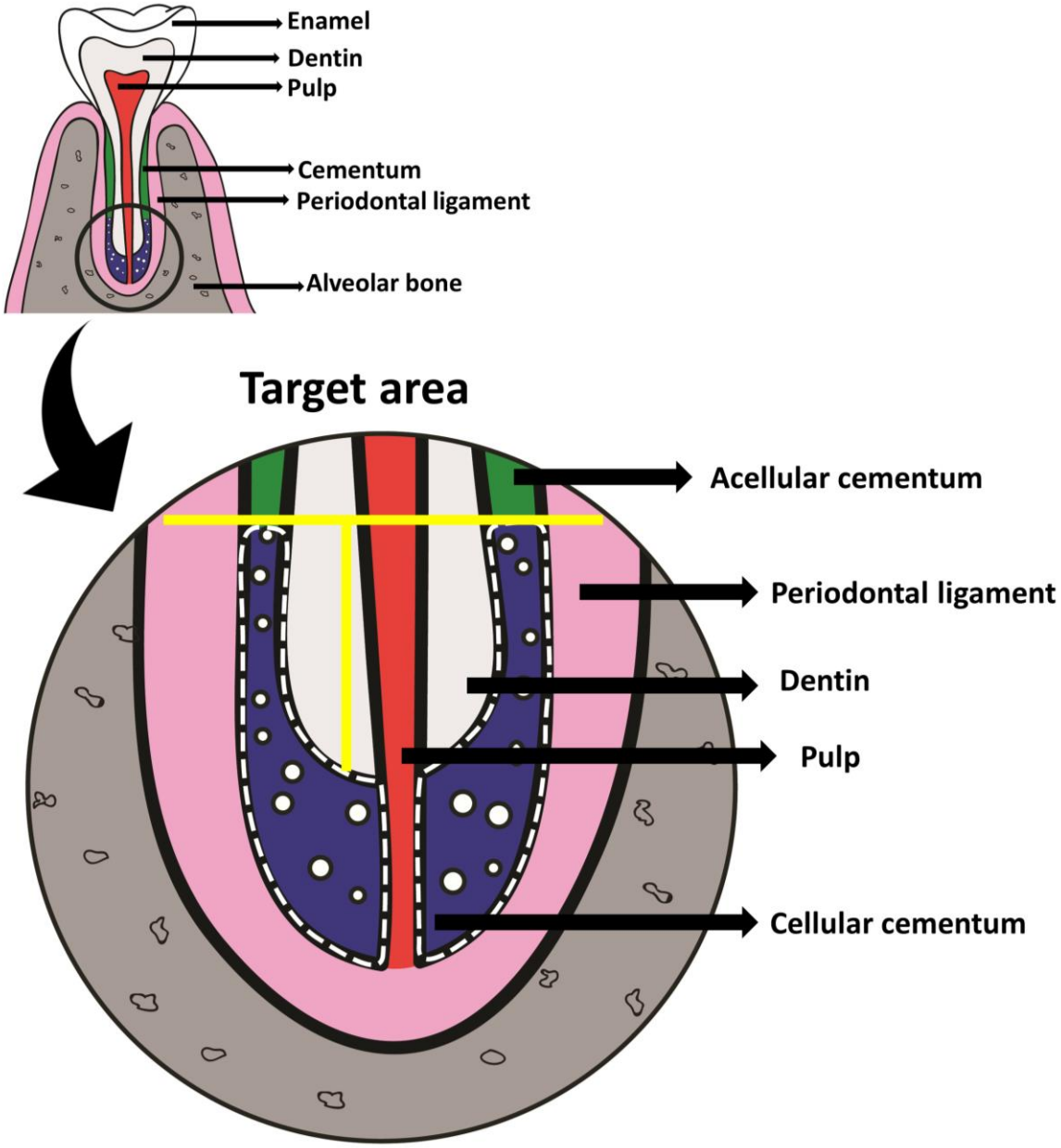

Figure 1. Schematic illustration of parameters assessed: cellular cementum area (white dotted line area), cementocyte number (white points), cellular cementum density (*e.g.,* number of cementocyte /cellular cementum area - purple area) and cementocyte body size (white points area). Yellow lines indicate as root dentin was measured (166 µm length) to guide the measurement on cellular cementum at the final portion of mandibular 1st molar.

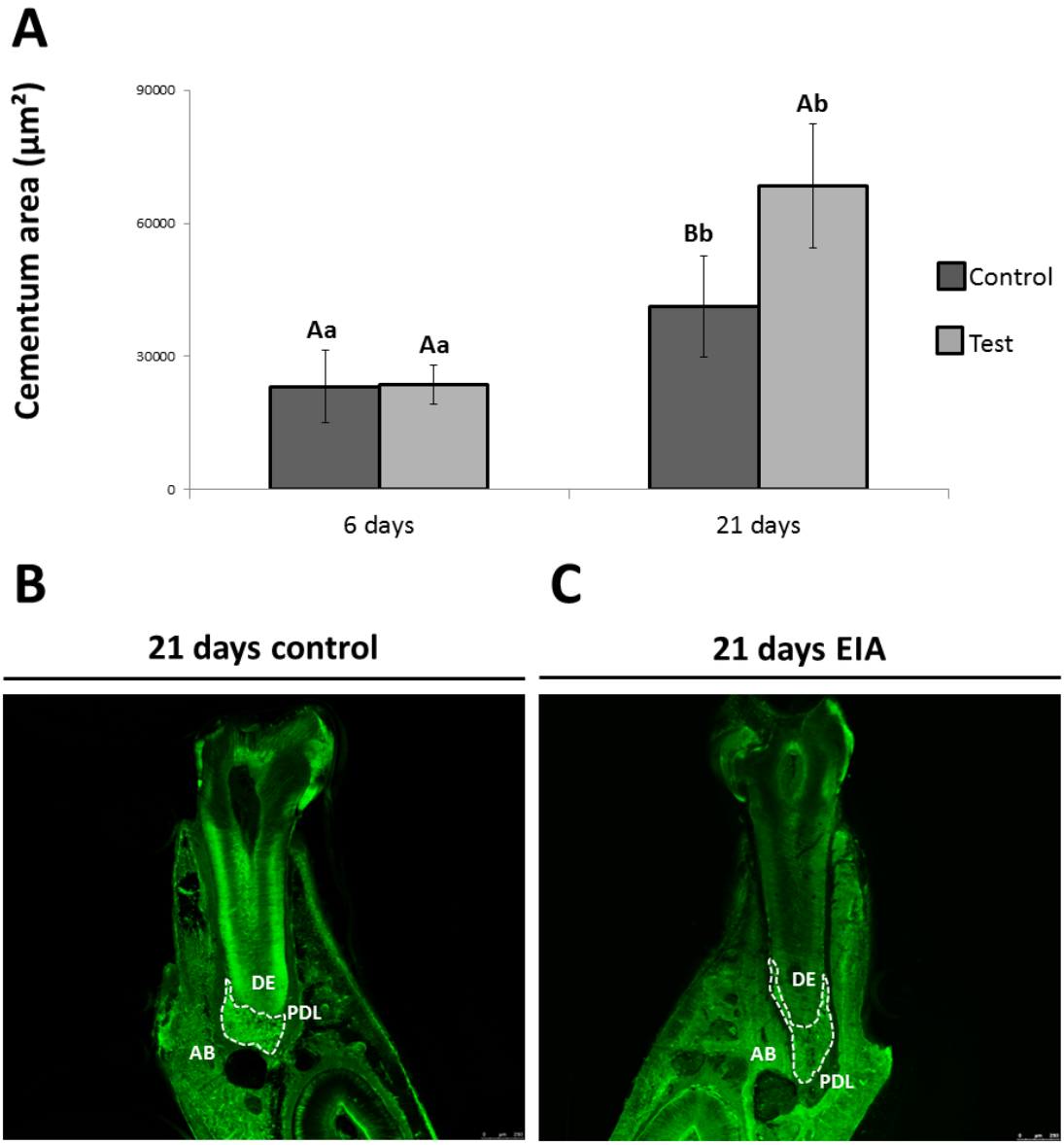

Figure 2. Increased cementum under experimentally-induced dental cementum apposition model. (A) During early cementum formation (6 days) mandibular first molars featured similar volume in both groups, whereas at 21 days was observed increased area between the groups, however experimental group presented higher values. (B, C) Confocal microscope images, labeling with Fluorescein Isothiocyanate x5 (FITC). In the apex region of first mandibular molars was observed increased dental cementum area in EIA group .

Abbreviations: CE- Dental Cementum; PDL - periodontal ligament; DE- dentin; AB- alveolar bone.

*Uppercase letter indicate comparisons over time in the same treatment group (intragroup). Lowercase letter indicate treatment comparison at each time point (intergroup). Same letters indicate no significant differences, while values with different letters were significantly different($p<0.05$) as tested by ANOVA followed by the Tukey test. Graphs show mean SD values for samples (n=5).

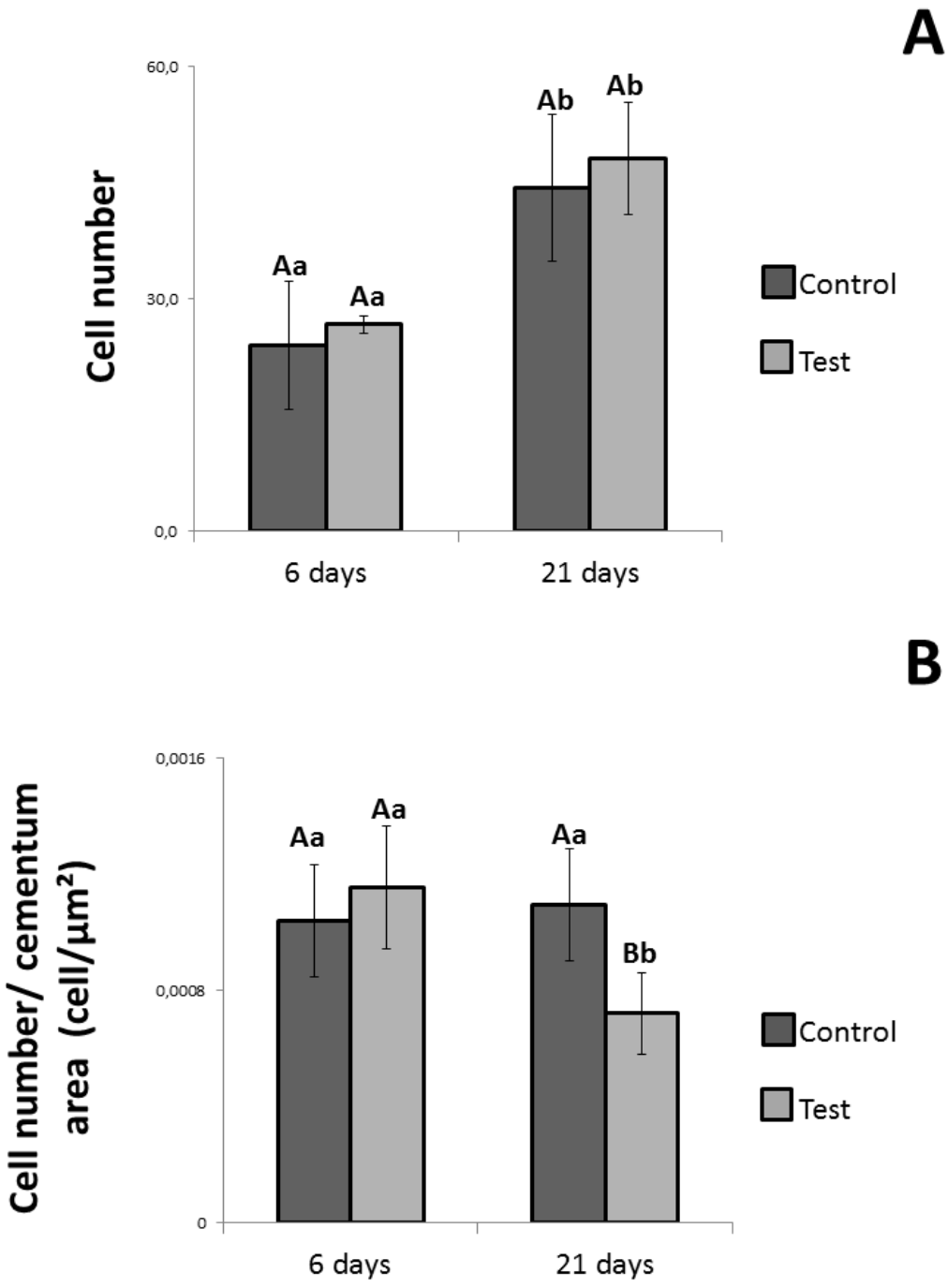

Figure 3. Experimentally-induced dental cementum apposition model does not affect the cementocyte number. (A) A quantitative approach was performed and cementocytes in a previously established area were counted. Increased cell counting at 21 days was observed, however experimental group value was not significantly different compared to control. (B) Relation of cementocyte number in pre-established area featured decreased significant difference under EIA conditions at 21 days. Suggesting that the amount of matrix formation is responsible for this inversely proportional relationship.

*Uppercase letter indicate comparisons over time in the same treatment group (intragroup). Lowercase letter indicate treatment comparison at each time point (intergroup). Same letters indicate no significant differences, while values with different letters were significantly different($p<0.05$) as tested by ANOVA followed by the Tukey test. Graphs show mean SD values for samples (n=5).

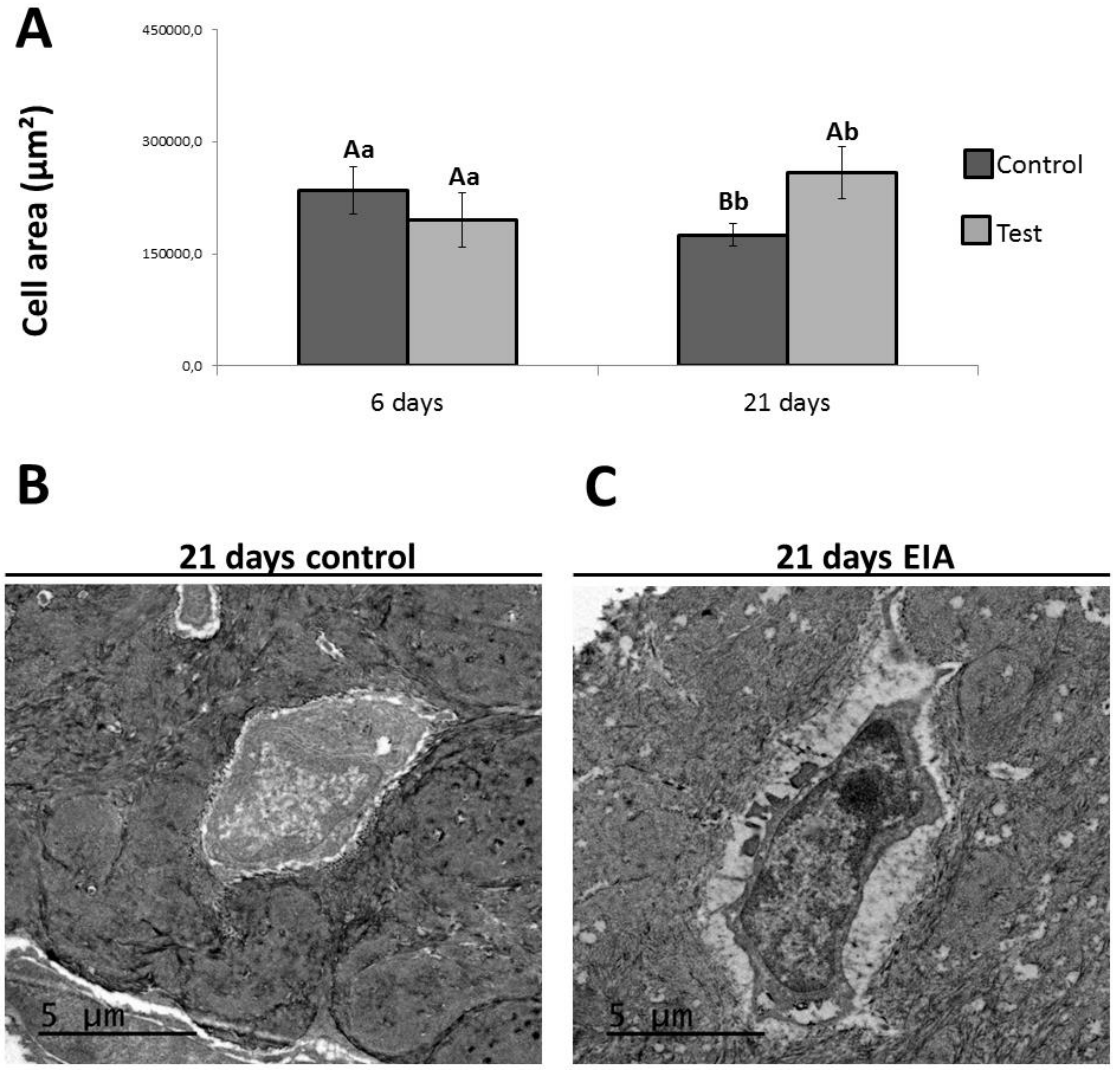

Figure 4. Increased cell area under experimentally-induced dental cementum apposition model. (A) Experimentally-induced dental cementum apposition model does not affect cell area at 6 days. On the other hand, 21 days experimental group featured significantly different value compared to control. (B, C) Transmission Electron Microscopy (TEM) images reinforces the findings after measurements in confocal images showing cementocytes increased cellular body area at 21 days x 10000.

*Uppercase letter indicate comparisons over time in the same treatment group (intragroup). Lowercase letter indicate treatment comparison at each time point (intergroup). Same letters indicate no significant differences, while values with different letters were significantly different($p<0.05$) as tested by ANOVA followed by the Tukey test. Graphs show mean SD values for samples (n=5).

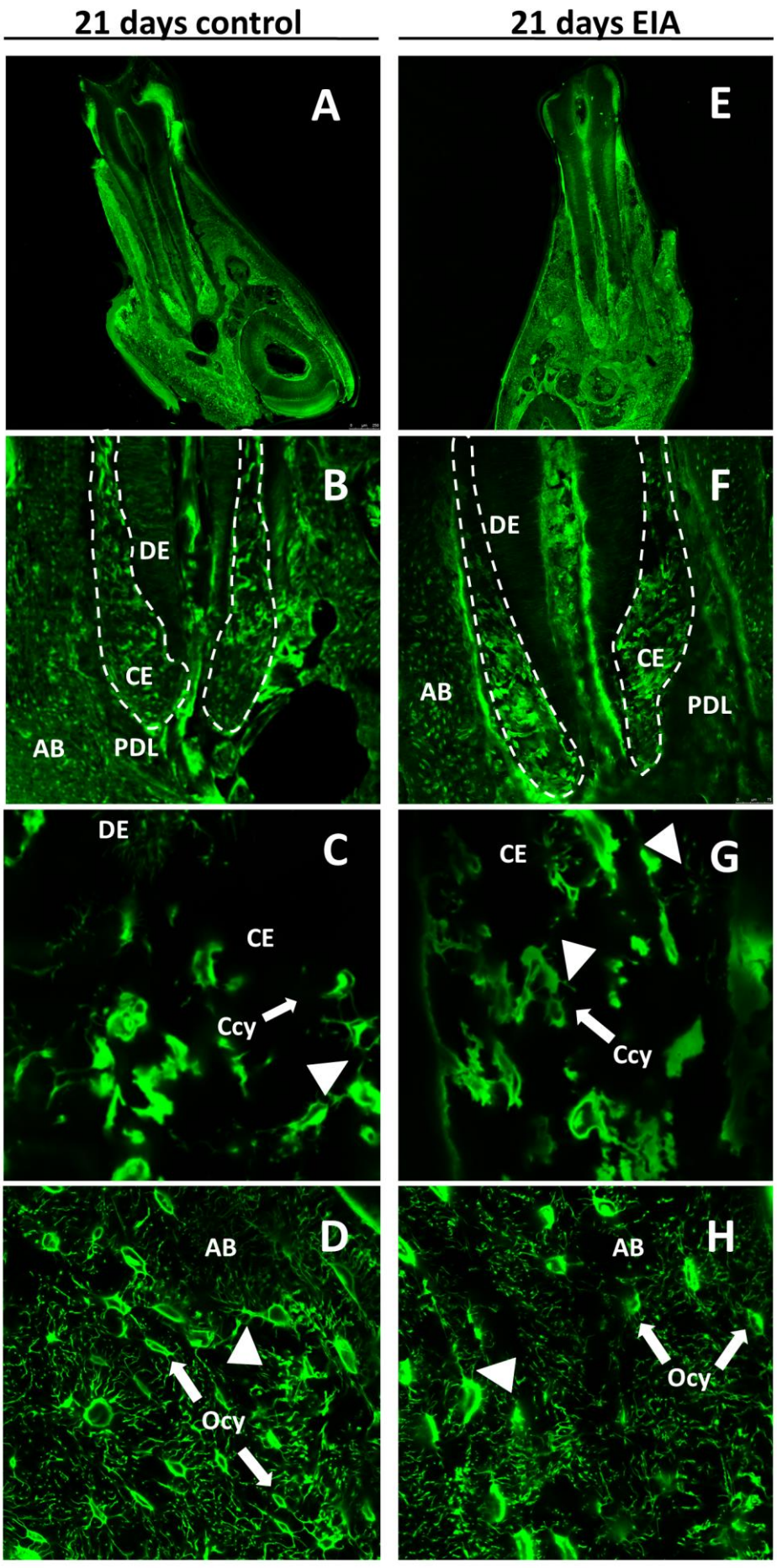
21 days control
21 days EIA
A
E
B
DE
CE
AB
PDL
F
DE
CE
AB
PDL
C
DE
CE
Ccy
G
CE
Ccy
D
AB
Ocy
H
AB
Ocy

Figure 5. Confocal microscope images, labeling with Fluorescein Isothiocyanate (FITC). (A, E) Section of mandibular first molar x5. (B, F) In the apex region was observed increased dental cementum area of EIA group x20. (C, G) Region of apical cementum at higher magnification, x63 (zoom 1.98). No difference related to dendritic processes both in number and in length was detected neither at 21 days experimental or control group. (D, H) Surrounding alveolar bone x63 (zoom 1.98). Photomicrographs revealed irregularly shaped cementocyte lacunae surrounded by few canaliculi compared to alveolar bone. Therefore, lacuno-canalicular network of the alveolar bone (AB) is better developed than in dental cementum (DC).

Arrows indicate cementocytes lacunae.
Head arrows indicate cellular projections

Abbreviations: CE - Dental Cementum; Ccy - cementocyte, PDL - periodontal ligament; DE - dentin; AB - alveolar bone; Ocy - Osteocyte.

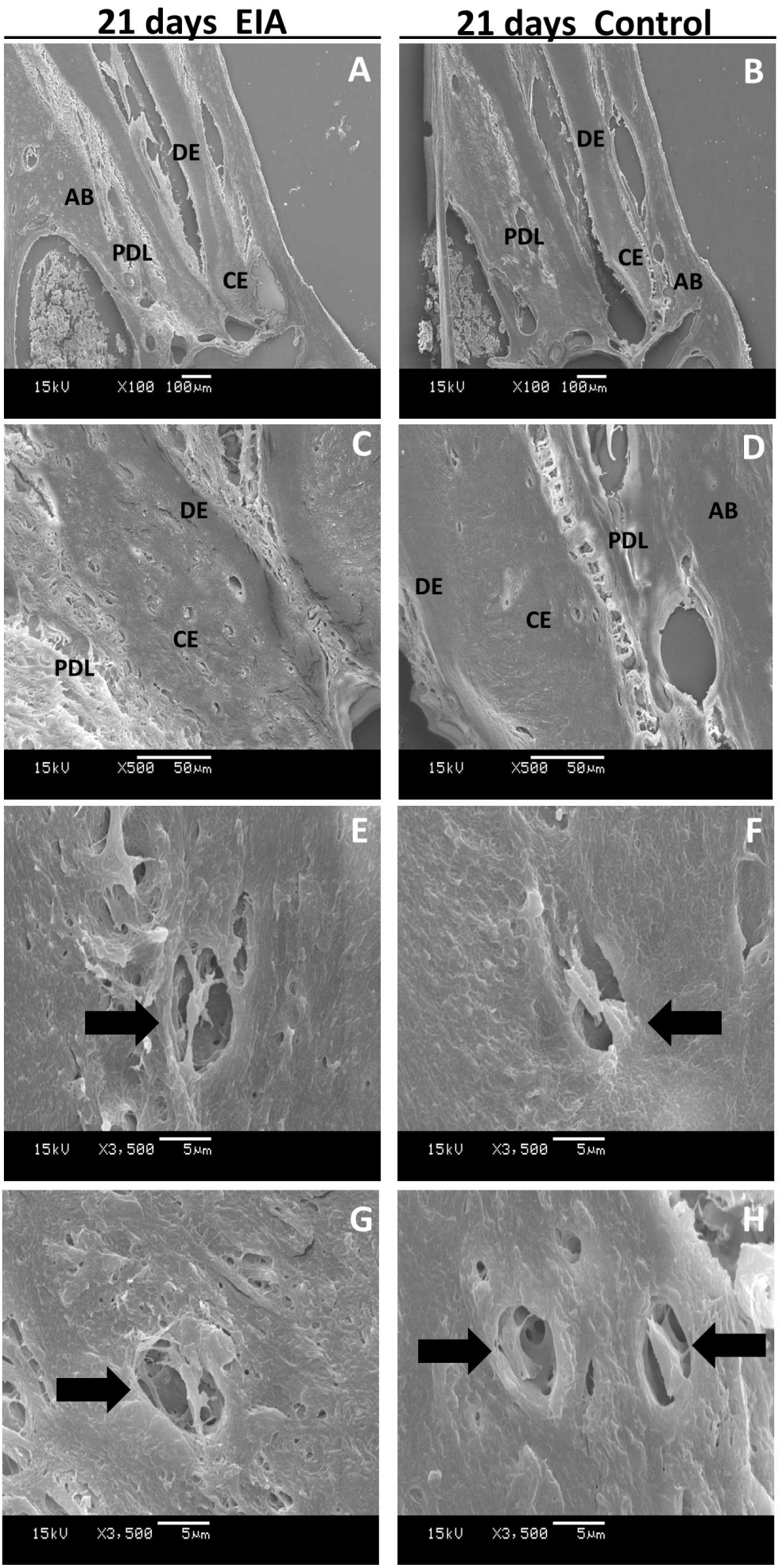
21 days EIA
21 days Control
A
DE
AB
PDL
CE
15kV X100 100µm
B
DE
PDL
CE
AB
15kV X100 100µm
C
DE
CE
PDL
15kV X500 50µm
D
AB
PDL
DE
CE
15kV X500 50µm
E
15kV X3,500 5µm
F
15kV X3,500 5µm
G
15kV X3,500 5µm
H
15kV X3,500 5µm

Figure 6. Scanning Electron Microscope show dental cementum morphology after experimentally-induced apposition (EIA) at 21 days. (A, B) Transverse section of mice mandibular molar x100. (C, D) Apical cementum at higher magnification x500. SEM images confirm that cementocytes occupying lacunae irregularly shaped and unevenly distributed in the matrix and numerous empty cementocyte lacunae. The two patterns observed were lacunae with cell remnants or empty lacunae. (E, F) Internal lacunae x3500. Cementum near the root surface presented lacunae larger in size in both groups. (G, H) External lacunae x3500. Representative SEM images show remnants cementocytes at 21 days.

Arrows indicate cementocytes lacunae.

Abbreviations: CE- Dental Cementum; PDL- periodontal ligament; DE- dentin; AB- alveolar bone.

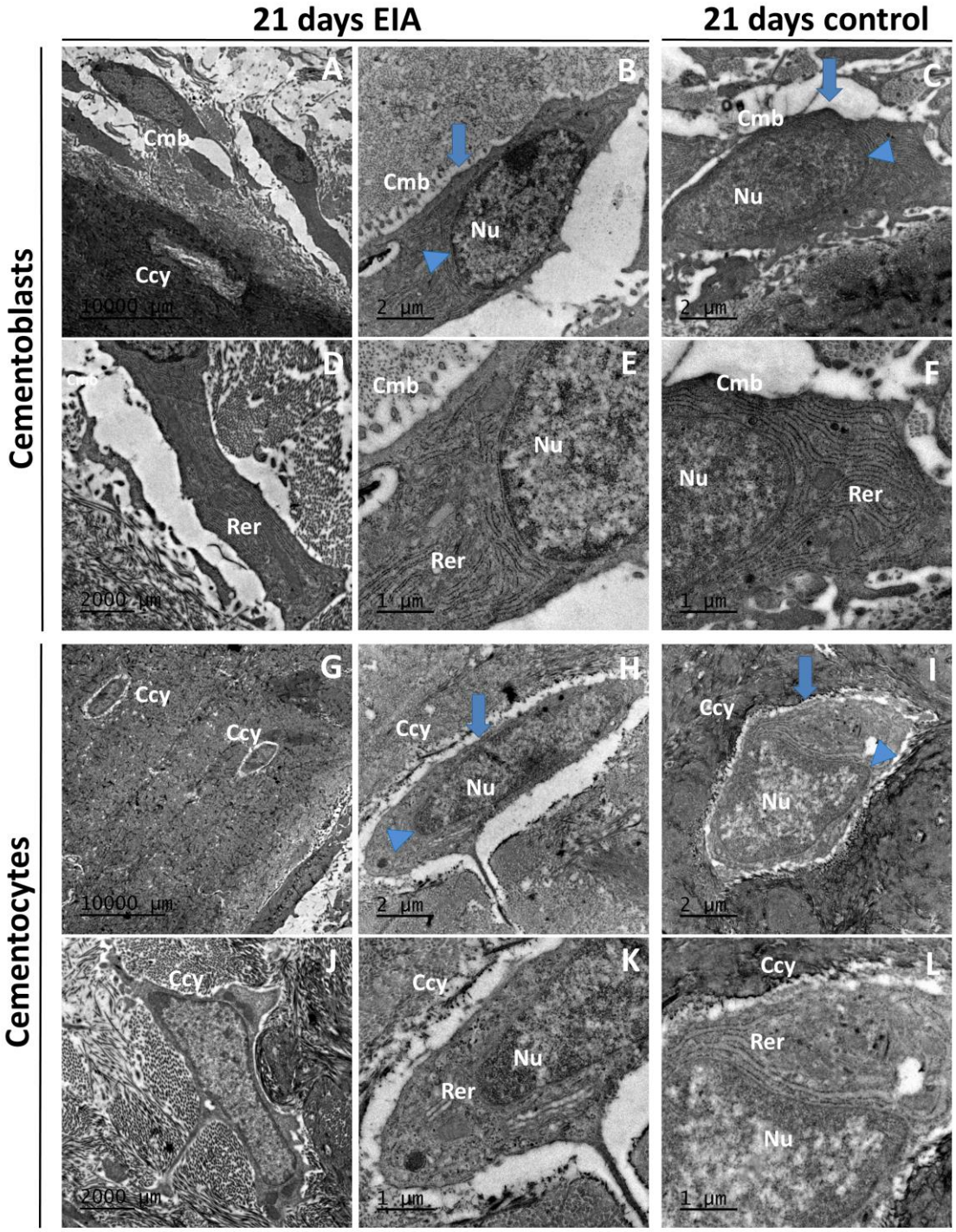
21 days EIA
21 days control
Cementoblasts
Cementocytes
A
B
C
D
E
F
G
H
I
J
K
L
Cmb
Nu
Ccy
Rer
10000 µm
2 µm
2000 µm
1 µm

Figure 7. Transmission Electron Microscopy **(**TEM) demonstrated changes on cellular ultrastructure of cementoblasts and cementocytes at the apical root of mandibular 1st molars in experimentally-induced apposition groups. (A) Cementoblasts at the cementum surface and cementocyte early trapped x3000. (B, C) At higher magnification it is possible to observe prominent and well-defined cell nuclei and abundant cytoplasm with rough endoplasmic reticulum (RER) and numerous ribosomes x12000. (C, F) The control group presented cementoblast with well-defined nuclei, intact nuclear membrane and well-developed rough endoplasmic reticulum (RER) x10000; x20000. (B, D, E) EIA group exhibited cementoblasts with well-defined nuclei and intact nuclear membrane. RER is more developed than the control group, indicating intense protein synthesis x10000; x20000. (G) Cementocytes trapped in the lacunae in different layers x3000. (H, I) Both the experimental and control groups' cementocytes presented smaller size compared to cementoblasts, defined nuclei with intact nuclear membrane and poorly developed rough endoplasmic reticulum X10000. (H) At 21 days we observed contracted cells within lacunae, while the control seemed to fill the lacunae even though cell size under experimentally-induced apposition were larger. Experimentally-induced apposition group featured increased cell area and nuclei x10000. (J) Cementum matrix presented radially arranged fibers bundles x10000. (K, L) TEM show a sharp difference in the cellular nuclei and cell proportion in the experimental group, where nuclei occupy substantial cell area whereas this ratio is lower in the control. Cementocyte present not only larger nuclei but also nuclei have more peripherally condensed chromatin and euchromatin spread in the nuclei than the controls after experimental induction x20000.

Abbreviations: Cmb- cementoblast; Ccy- cementocyte; Nu- nucleus; RER- rough endoplasmic reticulum.

3 CONCLUSÃO

Os dados mostraram que o modelo experimental foi eficiente em estimular a aposição de cemento, uma maior quantidade de área de tecido na região apical foi observada no grupo estimulado em relação ao controle em período mais tardio. Os achados morfológicos e ultraestruturais sugerem que os cementócitos, juntamente com cementoblastos, podem desempenhar um papel importante no controle da aposição experimental da matriz do cemento dental formado.

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ANEXO 1. Certificado de aprovação do Comitê de Ética no Uso de Animais – Universidade Estadual de Campinas/UNICAMP.

UNICAMP

CEUA/Unicamp

CERTIFICADO

Certificamos que o projeto intitulado **"Determinação do papel dos cementócitos na hemostasia do cemento dental"**, protocolo nº **3788-1**, sob a responsabilidade de **Prof. Dr. Francisco Humberto Nociti Júnior / Elis Janaina Lira dos Santos**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica ou ensino, encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais e do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal - CONCEA**, e foi aprovado pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas - CEUA/UNICAMP,** em **14 de maio de 2015**.

Vigência do projeto: 07/2015-02/2015
Espécie/Linhagem: Camundongos Heterogenéticos / Unib:SW (Swiss)
No. de animais: 50
Peso/Idade: 35 dias/10gr
Sexo: machos
Origem: CEMIB/UNICAMP

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio**.

Campinas, **14 de maio de 2015**.

Prof. Dr. Alexandre Leite Rodrigues de Oliveira
Presidente

Fátima Alonso
Secretária Executiva

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ANEXO 2. Comprovante de submissão do artigo.

Brazilian Journal of Oral Sciences <onbehalfof+brjorals+fop.unicamp.br@manuscriptcentral.com> Fev 13 em 2:35 PM

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13-Feb-2017

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